Cellular/Molecular

Increased Basolateral Amygdala Pyramidal Cell Excitability May Contribute to the Anxiogenic Phenotype Induced by Chronic Early-Life Stress

Andrew R. Rau,[1,2] Ann M. Chappell,[1] Tracy R. Butler,[3] Olusegun J. Ariwodola,[1] and Jeff L. Weiner[1]

[1]Department of Physiology and Pharmacology, School of Medicine, and [2]Neuroscience Graduate Program, Graduate School of Arts and Sciences, Wake Forest University, Winston-Salem, North Carolina 27157-1083, and [3]Department of Psychology, University of Dayton, Dayton, Ohio 45469

Adolescence represents a particularly vulnerable period during which exposure to stressors can precipitate the onset of psychiatric disorders and addiction. The basolateral amygdala (BLA) plays an integral role in the pathophysiology of anxiety and addiction. Acute and chronic stress promote increases in BLA pyramidal cell firing, and decreasing BLA excitability alleviates anxiety measures in humans and rodents. Notably, the impact of early-life stress on the mechanisms that govern BLA excitability is unknown. To address this gap in our knowledge, we used a rodent model of chronic early-life stress that engenders robust and enduring increases in anxiety-like behaviors and ethanol intake and examined the impact of this model on the intrinsic excitability of BLA pyramidal cells. Adolescent social isolation was associated with a significant increase in the intrinsic excitability of BLA pyramidal cells and a blunting of the medium component of the afterhyperpolarization potential, a voltage signature of calcium-activated potassium (K_{ca}) channel activity. Western blot analysis revealed reduced expression of small-conductance K_{ca} (SK) channel protein in the BLA of socially isolated (SI) rats. Bath application of a positive SK channel modulator (1-EBIO) normalized firing in *ex vivo* recordings from SI rats, and *in vivo* intra-BLA 1-EBIO infusion reduced anxiety-like behaviors. These findings reveal that chronic adolescent stress impairs SK channel function, which contributes to an increase in BLA pyramidal cell excitability and highlights BLA SK channels as promising targets for the treatment of anxiety disorders and comorbid addiction.

Key words: 1-EBIO; AHP; anxiety; basolateral amygdala; intrinsic excitability; stress

Significance Statement

Although anxiety disorders and alcohol addiction frequently co-occur, the mechanisms that contribute to this comorbidity are poorly understood. Here, we used a rodent early-life stress model that leads to robust and longlasting increases in behaviors associated with elevated risk of anxiety disorders and addiction to identify novel neurobiological substrates that may underlie these behaviors. Our studies focused on the primary output neurons of the basolateral amygdala, a brain region that plays a key role in anxiety and addiction. We discovered that early-life stress decreases the activity of a specific class of potassium channels and increases the intrinsic excitability of BLA neurons and present evidence that enhancing the function of these channels normalizes BLA excitability and attenuates anxiety-like behaviors.

Introduction

Exposure to stressors can trigger or exacerbate psychiatric illnesses like anxiety disorders and depression (Lupien et al., 2009; Cui et al., 2013; Koob, 2013). Moreover, there is compelling evidence that stress and anxiety play important roles in the development and maintenance of alcohol use disorders (Spanagel et al., 1995; Silberman et al., 2009; McCool et al., 2010). Adolescence is a particularly vulnerable developmental period during which exposure to stressors can precipitate the development of psychiatric disorders and associated behaviors (Heim and Nemeroff, 2001; Yorgason et al., 2013; Butler et al., 2014), as well as drug and alcohol addiction (Chappell et al., 2013; Balogun et al., 2014).

Received Jan. 29, 2015; revised May 27, 2015; accepted May 29, 2015.

Author contributions: A.R.R. and J.L.W. designed research; A.R.R., A.M.C., T.R.B., and O.J.A. performed research; A.R.R., A.M.C., and T.R.B. analyzed data; A.R.R. and J.L.W. wrote the paper.

This work was supported by National Institutes of Health Grants AA022046 (A.R.R.), AA021099 (J.L.W.), AA017531 (J.L.W.), and AA010422 (J.L.W.). We thank Dr. Brian McCool and Nancy Alexander for assistance with Western blotting and Eugenia Carter for assistance in generating the animals.

The authors declare no competing financial interests.

Correspondence should be addressed to Jeff L. Weiner, Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1083. E-mail: jweiner@wakehealth.edu.

DOI:10.1523/JNEUROSCI.0384-15.2015

 0270-6474/15/359730-11$15.00/0

Several brain regions contribute to the circuitry governing stress responsivity (Millan, 2003), and integral among these regions is the basolateral amygdala (BLA; Lalumiere, 2014). Indeed, acute and chronic stress is associated with increases in the intrinsic excitability (IE) of BLA pyramidal cells, the primary output neurons of this brain region (Rosenkranz et al., 2010; Hetzel and Rosenkranz, 2014). In addition, decreasing activity of these cells reduces anxiety and its associated behaviors in humans (McEwen and Olie, 2005) and anxiety-like behaviors in rodents (Silberman et al., 2010). Whereas it seems likely that early-life stress may lead to increased BLA excitability, no studies to date have directly explored the impact of adolescent stress on the IE of BLA pyramidal neurons.

To that end, we used a rodent adolescent social isolation model that engenders robust and enduring increases in many behavioral risk factors associated with anxiety disorders and addiction to determine whether a disruption in the IE of BLA pyramidal neurons is associated with the behavioral phenotype resultant of this model. We and others have shown that, relative to animals group housed during adolescence, rats deprived of peer social contact during this period exhibit increased anxiety-like behavior in adulthood on a number of well validated assays (Wright et al., 1991; Hall et al., 1998a; Hellemans et al., 2004; Lim et al., 2011; Chappell et al., 2013). Notably, some of these changes persist for months (Yorgason et al., 2013). Moreover, social isolation is also associated with significant and enduring increases in multiple measures of ethanol self-administration (Hall et al., 1998b; Deehan et al., 2007; McCool and Chappell, 2009; Chappell et al., 2013).

Neuronal IE is tightly regulated by numerous ion channels, including calcium-activated potassium (K_{ca}) channels. Given prior evidence that other stressors increase BLA excitability via a decrease in K_{ca} function (Rosenkranz et al., 2010; Hetzel and Rosenkranz, 2014), we hypothesized that adolescent social isolation may increase the IE of BLA pyramidal neurons via a similar mechanism. Using perforated-patch recording methods to preserve the intracellular milieu, we found that adolescent social isolation significantly enhances BLA pyramidal cell IE and decreases the amplitude of the medium afterhyperpolarization potential and expression of small-conductance K_{ca} (SK) channels. We also demonstrate that enhancing BLA SK channel function restores normal BLA pyramidal cell excitability in socially isolated (SI) animals and attenuates anxiety-like behaviors in commercially sourced rats with a social isolation-like phenotype.

Materials and Methods

Animals

All experiments were performed in accordance with the Wake Forest University Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals* set forth by the National Institutes of Health.

Adolescent social isolation

Male Long–Evans rats were sourced from a commercial supplier (Harlan) and assigned to one of the following groups.

Group housed. Rats arrived at postnatal day 21 (P21) and were housed in groups of four in large Plexiglas cages (33.0 × 59.7 cm; Nalgene) until animals were killed for *ex vivo* electrophysiology or Western blotting (P85–P109).

Socially isolated. Rats arrived at P21, remained group housed (GH; four per cage) in the large Plexiglas cages for 1 week, and were then individually housed in smaller Plexiglas cages (25.4 × 45.7 cm) for the remainder of the study (P85–P109). These animals were exposed to the same olfactory, visual, and auditory cues as group housed rats but were deprived of social contact with peer rats during this period.

Standard adult housed. Rats used for microinjection studies arrived at P63 and were singly housed in the small Plexiglas cages for the remainder of the study. Bilateral BLA cannulation surgery was performed at P71 and P72, and microinjection studies were performed at P79–P106. Standard adult animals used for electrophysiology arrived at P63 and received a mock cannulation surgery at P71 and P72 in which animals were sedated, but cannulation of the BLA was not performed. These animals were handled in a manner similar to that of subjects used for the behavioral studies, including restraint commensurate with microinjections, but did not receive behavioral testing. Electrophysiology was performed at P101–P115.

GH/SI animals were weighed and handled once per week, whereas standard adult rats were handled and weighed 5 d per week. All animals had *ad libitum* access to food and water throughout the study. Animals were maintained on the same light/dark cycle and ate the same diet.

Electrophysiology

After the induction of a deep anesthetic plane with halothane, rats were decapitated, and their brains were removed and placed into ice-cold artificial CSF (aCSF) consisting of (in mM) 124 NaCl, 3.3 KCl, 2.4 $MgCl_2$, 1.2 KH_2PO_4, 10 D-glucose, and 25 $NaHCO_3$, bubbled with 95% O_2 and 5% CO_2. Transverse slices containing the basolateral amygdala were cut at a thickness of 400 μm using a VT1000S Vibratome (Leica Microsystems). Incubation of slices occurred for at least 1 h at room temperature (21–23°C) in aCSF before experiments commenced.

Slices were transferred to a recording chamber and perfused with oxygenated, room-temperature aCSF at 2 ml/min. Filamented borosilicate glass capillary tubes (inner diameter, 0.86 μm) were pulled using a horizontal pipette puller (P-97; Sutter Instrument) to prepare recording electrodes. Perforated-patch recordings were acquired in current-clamp mode using an Axoclamp 2B amplifier, digitized (Digidata 1321A; Molecular Devices), and analyzed off-line using an IBM-compatible computer running pClamp 10.4 software (Molecular Devices).

All electrophysiological recordings and analyses took place with the experimenter blind to the housing condition of the animals. The gramicidin perforated-patch technique was used for all electrophysiological experiments. Gramicidin is a polypeptide antibiotic that perforates the membrane, forming pores that are selectively permeable to monovalent cations (Akaike and Harata, 1994). This allows for direct electrical access to the cell without substantial dialysis of the cytosol (Akaike, 1996; Kaczorowski et al., 2007). Gramicidin was diluted in dimethylsulfoxide (DMSO) to a stock concentration of 50 mg/ml. The stock solution was further diluted to a final concentration of 200 μg/ml in a patch-pipette solution containing (in mM) 135 KCl, 10 HEPES, 2 $MgCl_2$, 2 EGTA, and 0.5 $CaCl_2$, adjusted to pH 7.2 with KOH. The KCl–gramicidin solution was sonicated for 5 min at the beginning of each day and vortexed for 15–30 s before filling each electrode. No filtering was applied. Each electrode was backfilled with gramicidin-free KCl patch-pipette solution to avoid interference of the antibiotic with seal formation, and the remainder of the electrode was filled with the KCl–gramicidin solution. After forming a high-resistance seal (GΩ), the cell was held in current-clamp mode for 25–75 min until perforation occurred and access resistance stabilized. All cells were maintained at a membrane potential of −60 mV with direct current injection. To examine the effect of adolescent social isolation on IE, BLA pyramidal neurons were injected with hyperpolarizing and depolarizing current pulses ranging from −300 to 550 pA in 50 pA increments for 600 ms. Current injections were separated by a 15 s interstimulus interval. The number of action potentials evoked by each current injection was counted and averaged across five trials for each experimental condition (e.g., baseline, drug application). Resting membrane potential (RMP) was assessed at the beginning of the recording period after the stabilization of access resistance and was periodically monitored throughout the recording by momentarily relieving the direct current injection. Although there were no significant differences in RMP between groups or from drug application, any changes detected in RMP during individual recordings were offset with manual direct current injection to maintain cells at −60 mV. Thus, any changes in action potential generation were not attributable to hyperpolarization or depolarization of the neuron. For all but the first experiment, 100 μM

picrotoxin and 20 μM 6,7-dinitroquinoxaline (DNQX) were added to the superfusate to block $GABA_A$ and AMPA receptors, respectively.

Unless otherwise noted, all drugs were purchased from Sigma. The selective SK channel modulators 1-ethyl-2-benzimidazolinone (1-EBIO) and apamin were purchased from Tocris. 1-EBIO was prepared as a 100-fold concentrate in water and DMSO and applied directly into the perfusion chamber via calibrated syringe pumps (Razel Scientific Instruments). Bath DMSO concentration never exceeded 0.05%. Apamin was delivered at its final concentration via a separate aCSF container, and a multichannel valve controller (Warner Instruments) was used to toggle between solutions using solenoid valves (Farmington Engineering).

Western blotting

Slices were prepared in the same manner as for electrophysiology studies (400 μM). Slices were then further dissected to remove extraneous tissue resulting in a BLA-enriched amygdala fraction and frozen at −80°C until used. Ten micrograms of total protein were loaded per lane onto 4–20% Criterion TGX precast polyacrylamide gels, separated by electrophoresis, and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked with Tris-buffered saline (TBST) containing 5% nonfat milk (NFM). Blots were then incubated overnight at 4°C in TBST/1.0% NFM containing an anti-SK2 or anti-SK3 polyclonal rabbit antibody (each at 1:1000; Alomone Labs). Blots were exposed to a peroxidase-labeled goat anti-rabbit secondary antibody (1:3000; Sigma). Detection of a bound secondary antibody was performed using Super Signal West Dura Extended Duration Substrate enhanced chemiluminescence (Thermo Fisher Scientific). Blots were stripped and reprobed with the loading control anti-β-actin mouse monoclonal antibody (Sigma). For analysis, each band was calculated as percentage of β-actin using Image-Lab software (Bio-Rad) and normalized to the GH group.

Behavioral assays

Microinjection experiments were conducted on male Long–Evans rats ($n = 8$, ~350 g) as described previously (Silberman et al., 2010). Briefly, 26 gauge guide cannulae were bilaterally implanted to terminate 1 mm dorsal to the BLA (2.8 mm posterior from bregma, 6.2 mm ventral from bregma, and 5.0 mm lateral from midline). Rats were then allowed to recover for 1 week. Rats were individually housed throughout the duration of this experiment.

After the recovery period, a 3 d acclimation procedure was conducted to habituate the rats to the stimuli associated with microinjections and to reduce the stress associated with this procedure. Four days after this acclimation period, behavioral testing commenced using a randomized, Latin-square design (vehicle, 1-EBIO). 1-EBIO was aided into solution using (2-hydroxypropyl)-β-cyclodextrin (10% w/v; Sigma) in aCSF (Tocris) and injected at 10 μg per side. (2-Hydroxypropyl)-β-cyclodextrin (10% w/v) in aCSF was used for vehicle injections. 1-EBIO or vehicle was injected at a volume of 0.5 μl per side over 60 s. Injection cannulae were left in place for an additional 30 s to allow for diffusion of drug or vehicle, and behavioral tests were administered 5 min after injections. Two behavioral tests were used to assess the effect of intra-BLA 1-EBIO injections on anxiety-like behaviors. The first test used a standard elevated plus-maze (elevated 72.4 cm from the ground) with four radial arms (10.2 × 50.8 cm) wherein opposing arms are either enclosed in black polypropelyne walls (40.6 cm high; MED Associates) or open and illuminated by incandescent light (~55 fc). Rats were placed at the central junction, facing an open arm, and activity was monitored for 5 min. Anxiety-like behavior was assessed as the time spent on the open arms and the total entries into the open arms, whereas gross locomotor activity was quantified as the number of entries into the closed arms. The second test used was the open-field exploration assay. Rats were placed in the center of an acrylic plastic chamber (40.7 × 40.8 × 40.7 cm). Exploratory activity was collected in 5 min bins for 30 min. Anxiety-like behavior was assessed as the amount of time spent in the center of the open field (20.3 × 20.3 cm). Locomotor activity was assessed as the total distance traveled. All activity within the open-field chamber and the elevated plus-maze was video recorded and analyzed off-line using EthoVision (Noldus) tracking software. Trials on the elevated plus-maze were separated by 1 month, with open-field tests conducted during the intervening weeks. Under these experimental conditions, this separation avoids one-trial tolerance that may develop on this assay (Silberman et al., 2010; Skelly and Weiner, 2014). After the completion of behavioral testing, all rats were given a lethal dose of sodium pentobarbital to induce a deep, irreversible anesthetic plane. Once deeply anesthetized, rats were transcardially perfused with 3% PBS to the point of exsanguination, followed by 10% formalin in saline. Brains were carefully removed via craniotomy and stored in 10% formalin for at least 1 week. Brains were then sectioned at a thickness of 90 μm using a Leitz 1320 cryostat (Boyle Instruments), stained with cresyl violet, and examined using light microscopy to confirm cannula placement.

Statistics

Data are expressed as mean ± SEM throughout the text and figures. Paired and unpaired two-tailed *t* tests were used when comparing two groups. When multiple measures were compared between groups, two-way repeated-measures ANOVAs (RM AVOVAs) were used. Where noted, *post hoc* analysis was conducted using Bonferroni's multiple comparisons test. The minimal level of significance was set as $p < 0.05$ for all analyses. All statistical analyses were conducted using Prism version 6.0 (GraphPad Software).

Results

Adolescent social isolation increases the excitability of BLA pyramidal neurons

To determine whether adolescent social isolation increased BLA excitability, the frequency of action potentials in response to depolarizing current steps was measured from BLA pyramidal neurons. Gramicidin perforated-patch recordings were performed in current-clamp mode where 600 ms hyperpolarizing and depolarizing current steps were applied (−300 to 550 pA in 50 pA increments; 15 s interstimulus interval). Between current pulses, neurons were held at −60 mV with direct current injection. Pyramidal neurons were characterized as those cells that displayed spike-frequency adaptation and broad action potentials and lacked spontaneous discharge at resting membrane potential (Washburn and Moises, 1992). In the first set of experiments, recordings were made in the absence of $GABA_A$ and AMPA receptor blockers in the superfusate. Neurons from SI animals displayed significantly greater IE ($n_{GH} = 16$, $n_{SI} = 17$; $F_{current\ (11,341)} = 225.7$, $p < 0.0001$; $F_{housing\ (1,31)} = 4.319$, $p = 0.0461$; $F_{interaction\ (11,341)} = 5.467$, $p < 0.0001$; two-way RM ANOVA; Fig. 1*A*,*B*) as indicated by an increased number of action potentials over a range of current injections.

Action potential frequency is mediated in part by both voltage- and calcium-activated potassium (K_{ca}) channels (Armstrong and Hille, 1998; Adelman et al., 2012). Intracellular increases in calcium occurring during action potentials activate K_{ca} channels, resulting in potassium efflux and membrane hyperpolarization. This afterhyperpolarization potential (AHP) shifts the neuronal membrane potential farther from the action potential threshold, making subsequent action potentials less likely, and can serve as a signature of K_{ca} activity (Rosenkranz et al., 2010; Padula et al., 2015). The amplitude of both the slow AHP (sAHP) and the medium AHP (mAHP) was measured from traces with similar action potential frequency, as the amplitude of these potentials is dependent on the number of spikes (Abel et al., 2004). mAHPs were measured as the peak negative potential after the cessation of the current step, whereas sAHPs were quantified as the average voltage during a 50 ms time window 280 ms after the end of the current step (Fig. 1*F*; Santini et al., 2008). Both the sAHP ($n_{GH} = 16$, $n_{SI} = 17$; $p = 0.0370$, unpaired *t* test; Fig. 1*C*,*F*) and the mAHP ($n_{GH} = 16$, $n_{SI} = 17$; $p = 0.0360$, unpaired *t* test; Fig. 1*D*,*F*) were blunted in neurons recorded from SI rats compared with those from GH animals, consistent with a stress-

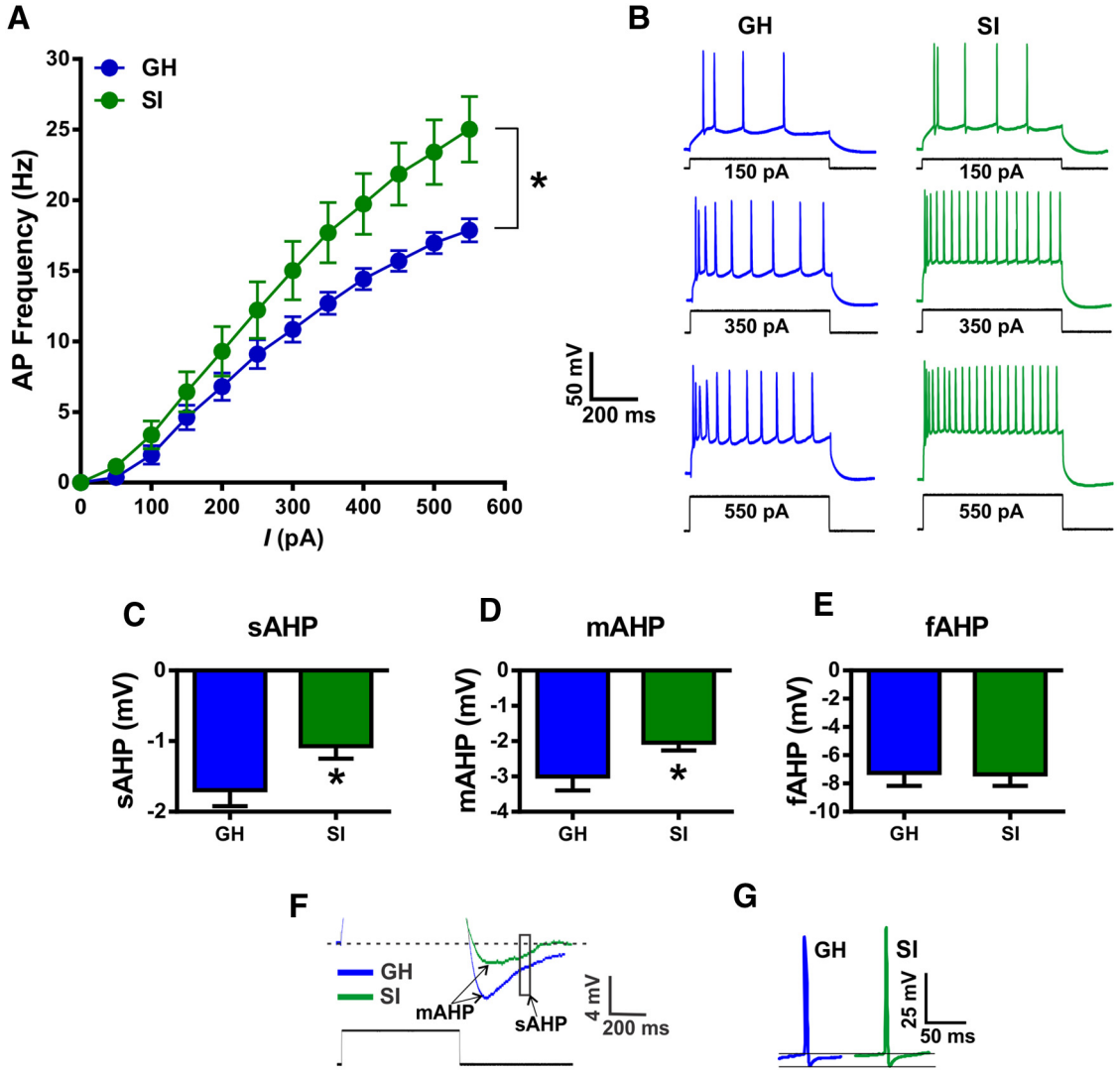

Figure 1. Adolescent social isolation is associated with an increase in the excitability of BLA pyramidal neurons. ***A***, Depolarizing current steps evoke significantly more action potentials in neurons recorded from SI animals compared with GH animals ($n_{GH} = 16, n_{SI} = 17$; $^*p < 0.05$, two-way RM ANOVA). ***B***, Representative voltage responses to 600 ms current pulses over a range of current intensities for recordings from GH and SI rats. ***C***, Group data showing that adolescent social isolation reduces the amplitude of the sAHP ($n_{GH} = 16, n_{SI} = 17$; $^*p < 0.05$, unpaired *t* test). ***D***, Group data indicating a significant effect of housing condition on the mAHP ($n_{GH} = 16, n_{SI} = 17$; $^*p < 0.05$, unpaired *t* test). ***E***, Group data showing that the fAHP is not modulated by social isolation ($n_{GH} = 16, n_{SI} = 17$; $p > 0.05$, unpaired *t* test). ***F***, Example traces showing the voltage response to a 600 ms depolarizing current step used to elicit a sAHP and a mAHP (spikes are truncated). The mAHP was quantified as the peak hyperpolarization, and the box indicates the window in which the sAHP was measured. The dashed line indicates the resting membrane potential of −60 mV. ***G***, Example traces showing the fAHP measured as the peak hyperpolarization relative to action potential threshold after a single action potential.

induced inhibition of K_{ca} channel activity (Rosenkranz et al., 2010). Fast AHPs (fAHPs) were quantified as the peak negative potential after the first action potential at the current step that elicited the fewest action potentials (Fig. 1*G*). No group differences were detected in the fAHP ($n_{GH} = 16, n_{SI} = 17; p = 0.9298$, unpaired *t* test; Fig. 1*E*,*G*).

Adolescent social isolation increases the intrinsic excitability of BLA pyramidal neurons independent of synaptic input

Action potential generation in response to depolarizing currents steps was again tested in BLA pyramidal neurons from both GH and SI rats. However, to block any influence of ongoing synaptic activity, 100 μM picrotoxin and 20 μM DNQX were added to the superfusate to block $GABA_A$ and AMPA receptors, respectively. Again, neurons recorded from rats reared in isolation displayed increased IE ($n_{GH} = 18, n_{SI} = 18$; $F_{current\ (11,374)} = 383.0, p < 0.0001$; $F_{housing\ (1,34)} = 4.155, p = 0.0493$; $F_{interaction\ (11,374)} = 3.086, p = 0.0005$, two-way RM ANOVA; Fig. 2*A*,*B*) and blunted mAHPs ($n_{GH} = 15, n_{SI} = 15; p = 0.0396$, unpaired *t* test; Fig. 2*D*). The amplitude of the sAHP ($n_{GH} = 16, n_{SI} = 17; p = 0.0910$, unpaired *t* test; Fig. 2*C*) and fAHP ($n_{GH} = 15, n_{SI} = 16; p = 0.8446$, unpaired *t* test; Fig. 2*E*) was not different between groups. Other measures of both passive and active membrane properties did not differ between housing groups (Table 1).

Positive modulation of BLA SK channels suppresses action potential frequency to a greater extent in neurons recorded from SI animals

The above experiments indicate that pyramidal neurons in the BLA of isolated animals exhibit a significant increase in action potential firing in response to depolarizing current injections. This increase in excitability was accompanied by reduced mAHP amplitude, indicative of reduced K_{ca} channel activity. If altered activity of BLA K_{ca} is fundamentally involved in stress-induced

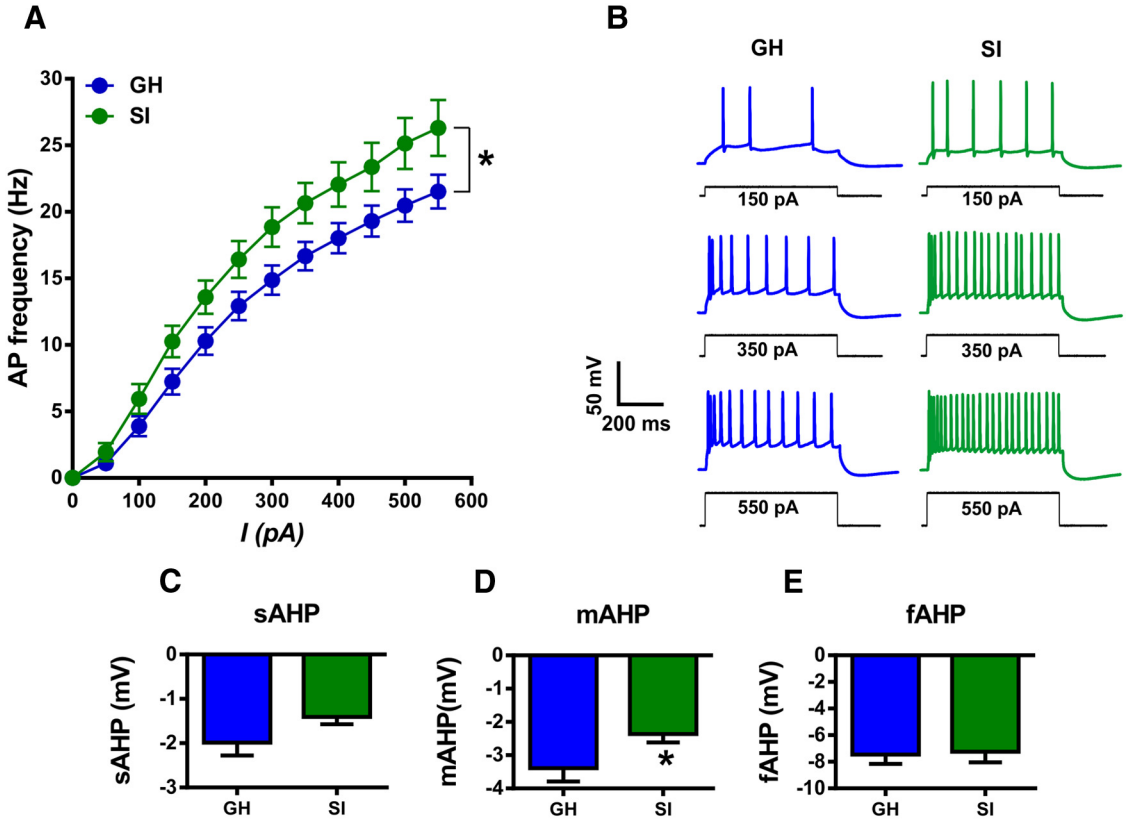

Figure 2. The adolescent social isolation-associated increase in BLA pyramidal neuron excitability is not dependent on synaptic transmission. ***A***, With synaptic transmission blocked, BLA pyramidal neurons recorded from SI rats fire significantly more action potentials in response to depolarizing current injections ($n_{GH} = 18$, $n_{SI} = 18$; $^*p < 0.05$, two-way RM ANOVA). ***B***, Representative voltage responses to 600 ms current pulses over a range of current intensities for recordings from GH and SI rats. ***C***, Group data showing that the sAHP is nonsignificantly decreased in recordings from SI animals ($n_{GH} = 16$, $n_{SI} = 17$; $p > 0.05$, unpaired *t* test). ***D***, Group data indicating a significant effect of adolescent housing condition on the amplitude of the mAHP ($n_{GH} = 15$, $n_{SI} = 15$; $^*p < 0.05$, unpaired *t* test). ***E***, Group data showing that adolescent housing condition does not affect the amplitude of the fAHP ($n_{GH} = 15$, $n_{SI} = 16$; $p > 0.05$, unpaired *t* test).

Table 1. Passive and active membrane properties of BLA pyramidal cells

	Group housed			Socially isolated				
	Baseline	1-EBIO	Apamin	Baseline	1-EBIO	Apamin	*F*	*p*
V_m (mV)	−67.65 ± 0.77	−67.07 ± 1.22	−67.18 ± 1.07	−66.71 ± 0.91	−66.73 ± 1.17	−65.9 ± 1.62	0.269	0.9288
R_{in} (MΩ)	166.6 ± 16.78	145.6 ± 10.17	129.3 ± 12.94	183.3 ± 22.61	147.0 ± 15.72	156.5 ± 22.59	1.243	0.2985
Rheobase (pA)	210.9 ± 17.12	212.1 ± 23.37	182.6 ± 21.18	190 ± 16.21	185.6 ± 25.08	154.7 ± 27.11	0.695	0.629
I_h (mV)	−4.98 ± 0.92	−3.91 ± 0.81	−4.71 ± 1.82	−4.47 ± 0.42	−5.52 ± 0.46	−4.86 ± 0.75	0.6154	0.6886
AP peak (mV)	78.59 ± 3.04	78.53 ± 3.06	74.42 ± 3.96	83.60 ± 1.65	85.96 ± 1.96	80.65 ± 3.50	1.884	0.1078
AP threshold (mV)	−44.95 ± 1.85	−43.41 ± 1.72	−43.42 ± 2.4	−47.31 ± 1.80	−44.43 ± 2.82	−47.07 ± 1.58	0.7791	0.5682

Shown is a summary of the effect of adolescent social isolation and SK channel modulators on other passive and active membrane properties of BLA pyramidal neurons. AP, Action potential.

increases in BLA pyramidal cell excitability, pharmacological enhancement of K_{ca} activity may mitigate the effects of chronic adolescent stress on BLA pyramidal cell excitability. Thus, we examined whether functional enhancement of SK channel activity with 1-EBIO, a drug that increases calcium sensitivity at the calmodulin-binding domain of SK channels (Pedersen et al., 1999; Zhang et al., 2012), differentially modulates action potential firing in SI and GH animals. Bath application of 300 μM 1-EBIO significantly suppressed action potential frequency in neurons from SI rats over a range of currents ($n = 15$; $F_{current\ (11,154)} = 80.30$, $p < 0.0001$; $F_{EBIO\ (1,14)} = 46.27$, $p < 0.0001$; $F_{interaction\ (11,154)} = 21.50$, $p < 0.0001$; two-way RM ANOVA; Fig. 3*B*) but was without effect in GH recordings ($n = 14$; $F_{current\ (11,143)} = 133.3$, $p < 0.0001$; $F_{EBIO\ (1,13)} = 3.271$, $p = 0.0937$; $F_{interaction\ (11,143)} = 1.450$, $p = 0.1569$; two-way RM ANOVA; Fig. 3*A*). After application of 1-EBIO, no housing group-related differences in either IE ($F_{housing\ (1,28)} = 0.02123$; $p = 0.8852$, two-way RM ANOVA; Fig. 4*A*,*B*, red traces) or mAHP ($n_{GH} = 15$, $n_{SI} = 15$; $p = 0.6706$, unpaired *t* test; Fig. 3*C*) were observed, suggesting that enhancing SK channel activity can normalize the increased BLA excitability that manifests after adolescent social isolation.

SK channel blockade differentially enhances firing in BLA pyramidal neurons from GH and SI animals

If increased IE of BLA pyramidal cells from SI animals reflect reduced SK channel activity, then blockade of these channels should have a smaller effect on firing in neurons from SI versus GH animals (Hopf et al., 2010). Apamin is a potent and selective SK channel blocker that reduces mAHP amplitude (Stocker et al., 1999) and can enhance action potential firing (Hopf et al., 2010). Although apamin (100 nM) enhanced firing in neurons recorded from both GH ($n = 10$; $F_{current\ (11,99)} = 168.0$, $p < 0.0001$; $F_{apamin\ (1,9)} = 9.845$, $p = 0.0120$; $F_{interaction\ (11,99)} = 12.67$, $p < 0.0001$; two-way

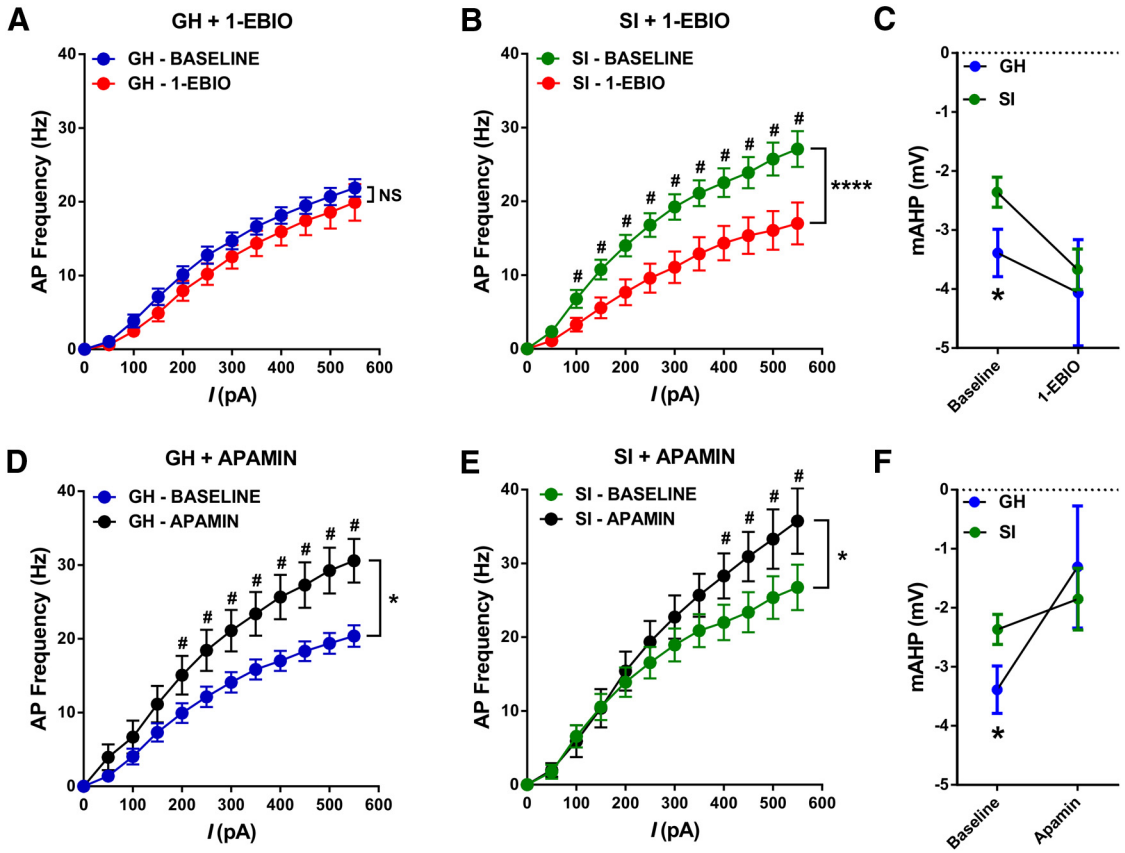

Figure 3. *Ex vivo* pharmacological modulation of BLA SK channels differentially affects action potential firing in recordings from SI animals compared with GH animals. ***A***, Bath application of the SK channel-positive modulator 1-EBIO does not attenuate firing in recordings from GH rats ($n = 14$; NS, not significant; two-way RM ANOVA). ***B***, Positive modulation of BLA SK channels significantly reduces action potential firing in recordings from SI rats ($n = 15$; $^{****}p < 0.0001$, two-way RM ANOVA; $^{\#}$significant difference from baseline, Bonferroni's multiple comparisons test). ***C***, 1-EBIO-positive modulation of SK channels normalizes mAHP amplitude between GH and SI recordings (baseline: $n_{GH} = 15$, $n_{SI} = 15$; $^{*}p < 0.05$, unpaired *t* test; 1-EBIO: $n_{GH} = 12$, $n_{SI} = 14$; $p > 0.05$, unpaired *t* test). ***D***, Blockade of SK channels with apamin increases action potential firing in recordings from GH rats ($n = 10$; $^{*}p < 0.0001$, two-way RM ANOVA; $^{\#}$significant difference from baseline, Bonferroni's multiple comparisons test). ***E***, Action potential firing is significantly increased after bath application of apamin in recordings from SI rats ($n = 9$; $^{*}p < 0.0001$, two-way RM ANOVA; $^{\#}$significant difference from baseline, Bonferroni's multiple comparisons test). ***F***, Apamin blockade of SK channels normalizes mAHP amplitude between the GH and SI groups (baseline: $n_{GH} = 15$, $n_{SI} = 15$; $^{*}p < 0.05$, unpaired *t* test; apamin: $n_{GH} = 10$, $n_{SI} = 9$; $p > 0.05$, unpaired *t* test).

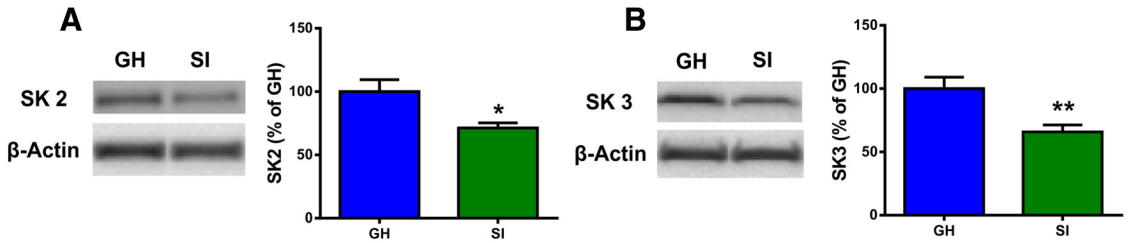

Figure 4. SI rats have reduced BLA SK2 and SK3 subunit protein expression compared with GH subjects. ***A***, Representative Western blots and group data, normalized to GH, illustrating reduced SK2 subunit protein in the BLA of SI rats ($n_{GH} = 8$, $n_{SI} = 8$; $^{*}p < 0.05$, unpaired *t* test). ***B***, Representative Western blots and group data, normalized to GH, illustrating reduced SK2 subunit protein expression in SI rats. ($n_{GH} = 8$, $n_{SI} = 8$; $^{**}p < 0.01$, unpaired *t* test).

RM ANOVA; Fig. 3*D*) and SI ($n = 9$; $F_{current\ (11,88)} = 67.87$, $p < 0.0001$; $F_{apamin\ (1,8)} = 9.323$, $p = 0.0157$; $F_{interaction\ (11,88)} = 7.23$, $p < 0.0001$; two-way RM ANOVA; Fig. 3*E*) animals, Bonferroni's *post hoc* tests revealed that apamin did increase firing over a greater range of injected currents in GH rats (significant difference from baseline in GH animals from 200 to 550 pA and in SI animals from 400 to 550 pA; Fig. 3*D*,*E*), suggesting that the baseline contribution of SK channels is greater in GH animals. The amplitude of the mAHP was blunted in both groups after apamin application and was no longer significantly different between groups ($n_{GH} = 10$, $n_{SI} = 9$; $p = 0.6135$, unpaired *t* test; Fig. 3*F*).

SK2 and SK3 subunit protein expression is reduced after adolescent social isolation

Three subtpyes of SK channels are expressed in the brain, and all three subtypes are highly enriched within the BLA (Stocker and Pedarzani, 2000; Gymnopoulos et al., 2014). We therefore followed up the electrophysiological experiments by using Western blots to determine whether there was a concurrent decrease in SK channel protein in the BLA of isolated animals. We chose to analyze the expression of the SK2 and SK3 subunits as these subunits are associated with the current that underlies the mAHP (Bond et al., 2004) and are altered in the nucleus accumbens and

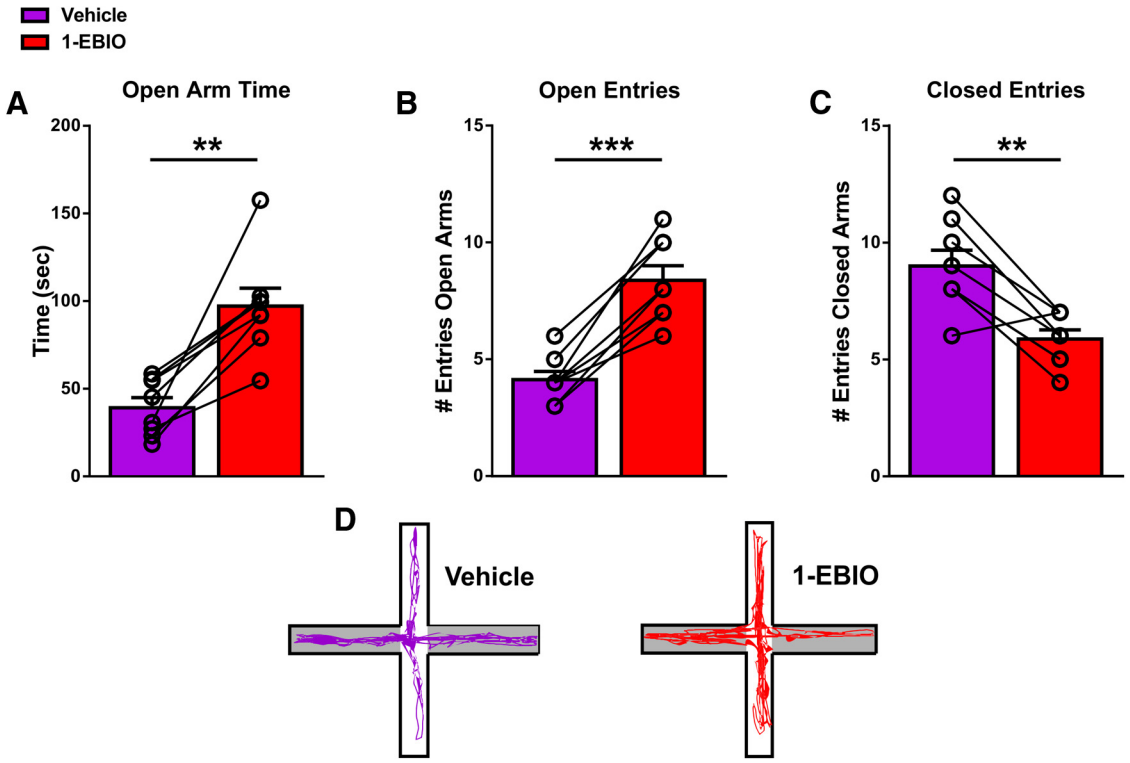

Figure 5. Intra-BLA infusion of a positive SK channel modulator reduces anxiety-like behavior on the elevated plus-maze. ***A***, Group (bars) and individual (open circle) data illustrating a significant increase in time spent on the open arms of the elevated plus-maze after bilateral microinjection of 1-EBIO (10 μg per side; $n = 8$; $^{**}p < 0.01$, paired *t* test). ***B***, Group and individual data indicating a significant 1-EBIO effect on entries into the closed arms of the elevated plus-maze ($n = 8$; $^{***}p < 0.001$, paired *t* test). ***C***, Group and individual data showing a significant reduction in closed-arm entries after microinjection of 1-EBIO ($n = 8$; $^{**}p < 0.01$, paired *t* test). ***D***, Activity tracks from representative rats during performance on the elevated plus-maze after vehicle (left) and 1-EBIO (right) microinjection; gray arms represent closed arms of the elevated plus-maze.

hippocampus after chronic ethanol exposure (Hopf et al., 2010; Mulholland et al., 2011; Padula et al., 2015). Both SK2 ($n_{GH} = 8$, $n_{SI} = 8$; $p = 0.0321$, unpaired *t* test; Fig. 4*A*) and SK3 ($n_{GH} = 8$, $n_{SI} = 8$; $p = 0.0051$, unpaired *t* test; Fig. 4*B*) subunit expression was significantly reduced in BLA-enriched tissue collected from SI rats relative to GH animals.

Activation of BLA SK channels decreases anxiety-like behaviors

Based on the preceding experiments, we hypothesized that pharmacological activation of BLA SK channels might attenuate some of the increases in behavioral risk factors (e.g., anxiety-like behaviors) associated with the adolescent social isolation procedure. To generate the phenotype observed in GH and SI animals, rats are minimally handled throughout adolescence (once per week for 7 weeks). The extensive handling required for brain region-specific microinjections precluded the use of SI and GH subjects for this experiment. However, we have previously reported that commercially sourced adult animals display a behavioral phenotype that is very similar to that of SI rats (Chappell et al., 2013), including an anxiogenic phenotype on the elevated plus-maze. Although these animals are also minimally handled during adolescence, we are able to acclimate them to microinjection procedures once they arrive in our laboratory. Bilateral delivery of 1-EBIO (10 μg per side) resulted in robust anxiolysis as evidenced by an increase in time spent on the open arms ($n = 8$; $p = 0.0012$, paired *t* test; Fig. 5*A*,*D*) and open-arm entries ($n = 8$; $p < 0.0001$, paired *t* test; Fig. 5*B*,*D*) on the elevated plus-maze. These changes were also associated with a significant reduction in closed-arm entries ($n = 8$; $p = 0.0022$, paired *t* test; Fig. 5*C*,*D*). Furthermore, 1-EBIO also reduced anxiety-like behavior on the open-field paradigm. After injection of 1-EBIO, rats spent significantly more time in the center of the open field compared with after vehicle injections ($n = 8$; $F_{\text{housing }(1,7)} = 7.685$; $p = 0.0276$, two-way RM ANOVA; Fig. 6*A*,*C*). Notably, no differences in overall locomotor activity were noted in this assay ($n = 8$; $F_{\text{housing }(1,7)} = 1.609$, $p = 0.2452$; $F_{\text{interaction }(5,35)} = 1.609$, $p = 0.9079$; two-way RM ANOVA; Fig. 6*B*,*C*). Since 1-EBIO significantly reduced IE in recordings from SI but not GH subjects, we next sought to determine whether BLA pyramidal cell excitability in commercially sourced rats was also sensitive to 1-EBIO modulation. We procured a separate cohort of commercially sourced rats and handled them in a manner similar to that used for the behavioral studies (see Materials and Methods). Bath application of 300 μM 1-EBIO significantly reduced the IE of BLA pyramidal cells ($n = 8$; $F_{\text{current }(11,77)} = 100.4$, $p < 0.0001$; $F_{\text{EBIO }(1,7)} = 11.65$, $p = 0.0112$; $F_{\text{interaction }(11,77)} = 8.051$, $p < 0.0001$; two-way RM ANOVA). To quantify the effect of 1-EBIO on IE in SI, GH, and commercially sourced rats, the number of action potentials generated over the full range of depolarizing current injections was fit with a linear slope to generate an input/output (I/O) slope (Hopf et al., 2010). This I/O relationship was compared between baseline and 1-EBIO time points in each cohort. 1-EBIO had no effect on the I/O slope in GH animals ($n = 14$; $p = 0.4045$; paired *t* test; Fig. 7*A*) but significantly inhibited this measure in both SI rats ($n = 15$; $p < 0.0001$, paired *t* test; Fig. 7*B*) and commercially sourced adult animals ($n = 8$; $p = 0.0051$, paired *t* test; Fig. 7*C*).

Discussion

The clinically important comorbidity between stress, anxiety disorders, and alcohol addiction has been well documented (Kushner et al., 2000; Grant et al., 2004; Koob and Le Moal, 2008; Silberman et al.,

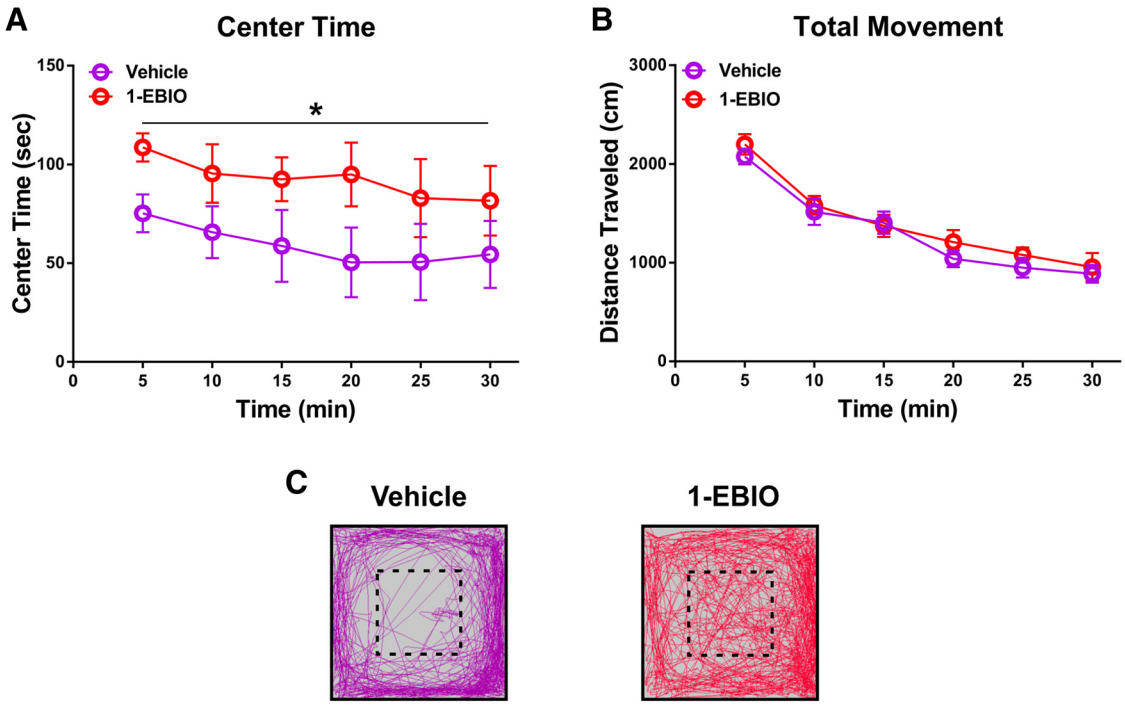

Figure 6. Intra-BLA infusion of a positive SK channel modulator is anxiolytic on the open-field test. ***A***, Time course demonstrating that rats spend significantly more time in the center area of an open field after positive modulation of BLA SK channels with 1-EBIO ($n = 8$; $^{*}p < 0.05$, two-way RM ANOVA). ***B***, Time course illustrating that bilateral microinjection in 1-EBIO (10 μg per side) into the BLA does not modulate locomotor behavior ($n = 8$; $p > 0.05$, two-way RM ANOVA). ***C***, Representative activity traces after vehicle (left) and 1-EBIO (right) microinjections on the open-field test. Dashed-line boxes indicate the center zone of the open field.

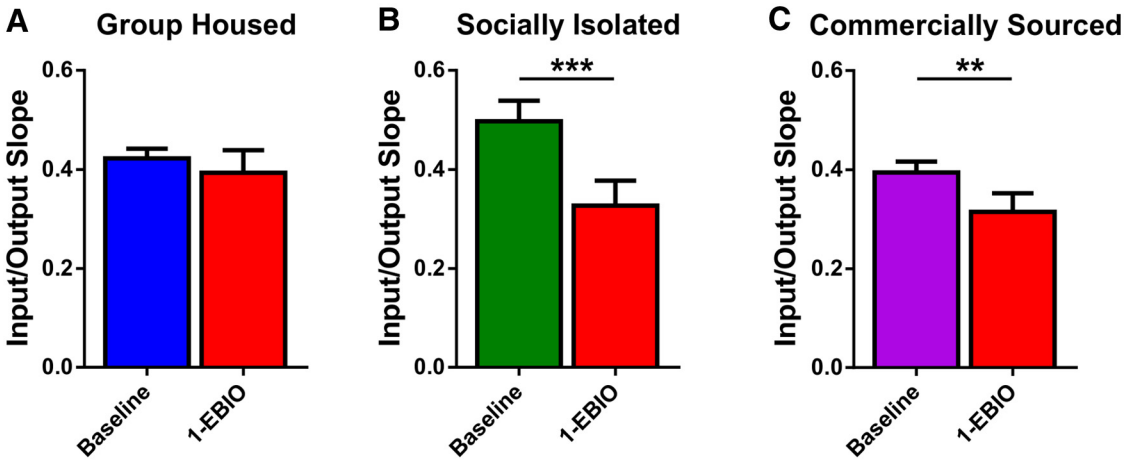

Figure 7. Positive *ex vivo* modulation of SK channels reduces intrinsic excitability of BLA pyramidal neurons in commercially sourced adult rats that show SI-like, anxiety-like behavior (Chappell et al., 2013). ***A***, Bath application of 1-EBIO (300 μM) does not significantly modulate the I/O slope in GH recordings ($n = 14$; $p > 0.05$, paired *t* test). ***B***, 1-EBIO reduces the I/O slope in recordings from SI rats ($n = 15$; $^{***}p < 0.0001$, paired *t* test). ***C***, Positive modulation of BLA SK channels reduces the I/O slope recorded from pyramidal cells in commercially sourced rats ($n = 8$; $^{**}p < 0.01$, paired *t* test).

2009; Koob, 2013). However, difficulties in developing animal models that foster robust increases in anxiety-like behaviors and other measures of addiction vulnerability have impeded advances in identifying neural substrates that may underlie these disorders. Here, we used a rodent adolescent social isolation procedure that engenders longlasting increases in a range of behaviors associated with increased risk of alcohol addiction (Hall et al., 1998a; Chappell et al., 2013; Yorgason et al., 2013; Butler et al., 2014) to examine the impact of early-life stress on the IE of BLA pyramidal cells. These neurons are thought to assign affective value to sensory signals with strong emotional valence (Janak and Tye, 2015; Namburi et al., 2015) and are the primary output neurons of this brain region that is known to play an integral role in the etiology of anxiety disorders and addiction (Stuber et al., 2011; Felix-Ortiz et al., 2013; Stamatakis et al., 2014). Using perforated-patch recording methods to preserve the integrity of the cytosolic milieu, we found that, relative to recordings from animals group housed in adolescence, BLA pyramidal cells from socially isolated rats displayed a significant increase in action potential firing in response to depolarizing current injections. This increase in excitability was associated with a decrease in the amplitude of the mAHP, suggesting that K_{ca} channel activity may be impaired in the BLA of SI animals, thus providing a pharmacological target to reverse the effects of chronic stress.

Plastic adaptations of neuronal membrane properties play an important role in learning and memory. However, maladaptive changes in membrane properties are observed in pathological conditions such as epilepsy (Bernard et al., 2004), drug addiction (Kourrich et al., 2015), and animal models of post-traumatic stress disorder (Santini et al., 2008; Criado-Marrero et al., 2014). Our data provide supporting evidence for the emerging hypothesis that K_{ca} channelopathy may underlie chronic stress-associated alterations in amygdala excitability (Rosenkranz et al., 2010; Hetzel and Rosenkranz, 2014). Although IE and AHP amplitude are regulated by a number of different ion channels, our data strongly suggest that SK channel dysregulation plays a central role in stress-induced increases in BLA pyramidal cell excitability. First, we observed reduced amplitude of the mAHP in recordings from SI animals. In the hippocampus, the mAHP is absent in SK2 null mice (Bond et al., 2004), and other studies have also linked the amplitude of mAHPs to the activity of SK channels (Rosenkranz et al., 2010; Padula et al., 2015). Second, our biochemical data reveal a robust reduction in SK2 and SK3 protein subunit expression in the amygdala of SI animals. Finally, we were able to normalize both mAHP amplitude and IE in SI recordings by pharmacologically enhancing SK channel function. K_{ca} channel function and expression is modulated by β-adrenoceptors (Faber et al., 2008), intracellular protein kinase activity (Pedarzani and Storm, 1993; Ren et al., 2006; Oh et al., 2009), glucocorticoids (Kye et al., 2007), and neuronal activity (Lin et al., 2010). It is plausible that stress-induced alterations in any of these factors could drive the modulation of SK channels observed in this study. Indeed, BLA adrenergic (Gilpin and Koob, 2010; Silberman et al., 2010), and protein kinase-dependent (Christian et al., 2012; Cruz et al., 2012), processes have all been implicated in stress–ethanol interactions, and modulators of these systems are indicated as potential targets for the treatment of both anxiety disorders and ethanol addiction (Sellers et al., 1977; Thorsell et al., 2007; Cui et al., 2013; Skelly and Weiner, 2014).

It is possible that the reduced amplitude of the sAHP may also contribute to the increased IE of BLA pyramidal cells in SI animals. The molecular identity of the channels underlying the sAHP are still unknown (Adelman et al., 2012); however, when we blocked SK channels with apamin, IE was normalized between groups. If the apamin-insensitive sAHP was driving the increased IE in SI animals, group differences would have been expected to remain during this experiment. Another alternative explanation for our results would be decreased activity of voltage-gated calcium channels, or decreased functional coupling between these calcium channels and K_{ca} channels. Although our data cannot definitively rule out these possibilities, the observed reduction in SK channel protein in SI BLA tissue is supportive of our hypothesis that K_{ca} channelopathy contributes to the increase in IE seen in SI animals. It is also possible that environmental conditions resulting in increased anxiety-like behavior alter the sensitivity of SK channels to positive allosteric modulation by 1-EBIO. Indeed, exposure to stress or drugs of abuse can alter allosteric interactions between drugs and their receptors (Deutsch et al., 1994; Kang et al., 1998).

We hypothesize that the ability of 1-EBIO to attenuate firing in SI, but not GH, animals, despite a significant reduction in channel expression in SI subjects, may be attributable to a ceiling effect. Basal SK channel activity in GH animals may be so high that it occludes the ability of 1-EBIO to further enhance mAHPs and reduce firing. In contrast, since SK channel activity appears to be compromised in SI animals, positive allosteric enhancement of these channels can augment mAHP amplitude and decrease the firing rate of BLA pyramidal neurons. Indeed, in other studies where SK channel activity and expression is reduced, positive modulators of SK channels also significantly reduced firing in groups exhibiting compromised SK channel activity but not in control subjects (Hopf et al., 2010, 2011b; Rosenkranz et al., 2010).

Chronic ethanol exposure also results in many of the behavioral and physiological phenotypes observed after chronic stress (McCool et al., 2010; Lopez and Becker, 2014), suggesting that environmental and ethanol-associated stressors may impinge on common physiological targets. Interestingly, chronic ethanol administration has also been associated with increases in IE of dorsal raphe (Lowery-Gionta et al., 2014), nucleus accumbens (Hopf et al., 2010), putamen (Cuzon Carlson et al., 2011), bed nucleus of the stria terminalis (Marcinkiewcz et al., 2014), and hippocampal (Mulholland et al., 2011) neurons. Meanwhile, the genes encoding for SK channels are altered in the BLA of alcoholics (Ponomarev et al., 2012) and in the nucleus accumbens of mice exposed to chronic ethanol (Padula et al., 2015). These data, taken together with our current findings, suggest that increases in neuronal firing along with SK channelopathy in many of the brain regions associated with addiction and stress responsivity, likely represent common neurobiological substrates that contribute to the deleterious behavioral sequelae associated with chronic stress or alcohol exposure.

BLA pyramidal neurons play a critical role in the processing of emotional information by assigning value to sensory cues and transmitting this signal onto efferent structures to regulate behavioral output in an adaptive manner (Tye and Janak, 2007; Namburi et al., 2015). As such, changes in the firing frequency of these cells can have dramatic behavioral outcomes and may contribute to the phenotype associated with the adolescent social isolation model. Increased excitability of BLA pyramidal neurons would likely facilitate activity in downstream regions such as the prefrontal cortex, central amygdala, hippocampus, and nucleus accumbens. Indeed, artificially increasing the activity of BLA pyramidal cells *in vivo* can produce a range of behaviors consistent with those observed in this model, including anxiety-like (Tye et al., 2011; Felix-Ortiz et al., 2013) and addiction-like (Stuber et al., 2011) behaviors.

The present findings demonstrate that adolescent social isolation results in a significant increase in the IE of BLA pyramidal neurons, likely mediated by a concurrent decrease in SK channel function and expression. Given the strong link between elevated BLA neuronal excitability and anxiety, this decrease in SK channel activity likely represents a critical neurobiological substrate that contributes to the longlasting increases in anxiety-like behaviors that manifest after the adolescent social isolation procedure. Indeed, our *ex vivo* electrophysiology experiments suggest that positive modulation of BLA SK channels can restore normal BLA IE in pyramidal cell recordings from SI animals. Consistent with this, we also provide evidence from studies in commercially sourced adult animals that exhibit an SI-like behavioral phenotype (Chappell et al., 2013), that intra-BLA infusion of this drug significantly reduces anxiety-like behaviors on two well validated behavioral assays. Importantly, we also confirmed that 1-EBIO significantly reduced BLA pyramidal cell excitability in recordings from commercially sourced rats, as observed in SI, but not in GH, subjects. Thus, in animals that exhibit an anxiogenic phenotype and a preservation of the ex vivo sensitivity to 1-EBIO inhibition of BLA IE, microinjection of this drug into the BLA significantly attenuates anxiety-like behaviors, providing initial

evidence that enhancement of BLA SK channels may represent an effective means of reducing anxiety and addiction-associated behaviors.

Collectively, these results demonstrate that early-life stress leads to a significant decrease in SK channel expression and activity and an increase in the IE of BLA pyramidal cells. Our findings, convergent with those of others (Rosenkranz et al., 2010; Hopf et al., 2011a,b; Atchley et al., 2012; Mulholland, 2012; Padula et al., 2015), contribute to a growing body of literature indicating that SK channels represent promising targets for the development of novel treatments for stress-associated anxiety disorders and comorbid addiction.

References

Abel HJ, Lee JC, Callaway JC, Foehring RC (2004) Relationships between intracellular calcium and afterhyperpolarizations in neocortical pyramidal neurons. J Neurophysiol 91:324–335. CrossRef Medline

Adelman JP, Maylie J, Sah P (2012) Small-conductance Ca2+-activated K+ channels: form and function. Annu Rev Physiol 74:245–269. CrossRef Medline

Akaike N (1996) Gramicidin perforated patch recording and intracellular chloride activity in excitable cells. Prog Biophys Mol Biol 65:251–264. CrossRef Medline

Akaike N, Harata N (1994) Nystatin perforated patch recording and its applications to analyses of intracellular mechanisms. Jpn J Physiol 44:433–473. CrossRef Medline

Armstrong CM, Hille B (1998) Voltage-gated ion channels and electrical excitability. Neuron 20:371–380. CrossRef Medline

Atchley D, Hankosky ER, Gasparotto K, Rosenkranz JA (2012) Pharmacological enhancement of calcium-activated potassium channel function reduces the effects of repeated stress on fear memory. Behav Brain Res 232:37–43. CrossRef Medline

Balogun O, Koyanagi A, Stickley A, Gilmour S, Shibuya K (2014) Alcohol consumption and psychological distress in adolescents: a multi-country study. J Adolesc Health 54:228–234. CrossRef Medline

Bernard C, Anderson A, Becker A, Poolos NP, Beck H, Johnston D (2004) Acquired dendritic channelopathy in temporal lobe epilepsy. Science 305:532–535. CrossRef Medline

Bond CT, Herson PS, Strassmaier T, Hammond R, Stackman R, Maylie J, Adelman JP (2004) Small conductance Ca^{2+}-activated K^+ channel knock-out mice reveal the identity of calcium-dependent afterhyperpolarization currents. J Neurosci 24:5301–5306. CrossRef Medline

Butler TR, Ariwodola OJ, Weiner JL (2014) The impact of social isolation on HPA axis function, anxiety-like behaviors, and ethanol drinking. Front Integr Neurosci 7:102. CrossRef Medline

Chappell AM, Carter E, McCool BA, Weiner JL (2013) Adolescent rearing conditions influence the relationship between initial anxiety-like behavior and ethanol drinking in male Long–Evans rats. Alcohol Clin Exp Res 37 [Suppl 1]:E394–E403.

Christian DT, Alexander NJ, Diaz MR, Robinson S, McCool BA (2012) Chronic intermittent ethanol and withdrawal differentially modulate basolateral amygdala AMPA-type glutamate receptor function and trafficking. Neuropharmacology 62:2430–2439. CrossRef Medline

Criado-Marrero M, Santini E, Porter JT (2014) Modulating fear extinction memory by manipulating SK potassium channels in the infralimbic cortex. Front Behav Neurosci 8:96. CrossRef Medline

Cruz MT, Herman MA, Kallupi M, Roberto M (2012) Nociceptin/orphanin FQ blockade of corticotropin-releasing factor-induced gamma-aminobutyric acid release in central amygdala is enhanced after chronic ethanol exposure. Biol Psychiatry 71:666–676. CrossRef Medline

Cui C, Noronha A, Morikawa H, Alvarez VA, Stuber GD, Szumlinski KK, Kash TL, Roberto M, Wilcox MV (2013) New insights on neurobiological mechanisms underlying alcohol addiction. Neuropharmacology 67:223–232. CrossRef Medline

Cuzon Carlson VC, Seabold GK, Helms CM, Garg N, Odagiri M, Rau AR, Daunais J, Alvarez VA, Lovinger DM, Grant KA (2011) Synaptic and morphological neuroadaptations in the putamen associated with long-term, relapsing alcohol drinking in primates. Neuropsychopharmacology 36:2513–2528. CrossRef Medline

Deehan GA Jr, Cain ME, Kiefer SW (2007) Differential rearing conditions alter operant responding for ethanol in outbred rats. Alcohol Clin Exp Res 31:1692–1698. CrossRef Medline

Deutsch SI, Rosse RB, Mastropaolo J (1994) Environmental stress-induced functional modification of the central benzodiazepine binding site. Clin Neuropharmacol 17:205–228. CrossRef Medline

Faber ES, Delaney AJ, Power JM, Sedlak PL, Crane JW, Sah P (2008) Modulation of SK channel trafficking by beta adrenoceptors enhances excitatory synaptic transmission and plasticity in the amygdala. J Neurosci 28:10803–10813. CrossRef Medline

Felix-Ortiz AC, Beyeler A, Seo C, Leppla CA, Wildes CP, Tye KM (2013) BLA to vHPC inputs modulate anxiety-related behaviors. Neuron 79:658–664. CrossRef Medline

Gilpin NW, Koob GF (2010) Effects of β-adrenoceptor antagonists on alcohol drinking by alcohol-dependent rats. Psychopharmacology (Berl) 212:431–439. CrossRef Medline

Grant BF, Stinson FS, Dawson DA, Chou SP, Dufour MC, Compton W, Pickering RP, Kaplan K (2004) Prevalence and co-occurrence of substance use disorders and independent mood and anxiety disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. Arch Gen Psychiatry 61:807–816. CrossRef Medline

Gymnopoulos M, Cingolani LA, Pedarzani P, Stocker M (2014) Developmental mapping of small-conductance calcium-activated potassium channel expression in the rat nervous system. J Comp Neurol 522:1072–1101. CrossRef Medline

Hall FS, Huang S, Fong GW, Pert A, Linnoila M (1998a) Effects of isolation-rearing on locomotion, anxiety and responses to ethanol in Fawn Hooded and Wistar rats. Psychopharmacology (Berl) 139:203–209. CrossRef Medline

Hall FS, Huang S, Fong GW, Pert A, Linnoila M (1998b) Effects of isolation-rearing on voluntary consumption of ethanol, sucrose and saccharin solutions in Fawn Hooded and Wistar rats. Psychopharmacology (Berl) 139:210–216. CrossRef Medline

Heim C, Nemeroff CB (2001) The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry 49:1023–1039. CrossRef Medline

Hellemans KG, Benge LC, Olmstead MC (2004) Adolescent enrichment partially reverses the social isolation syndrome. Brain Res Dev Brain Res 150:103–115. CrossRef Medline

Hetzel A, Rosenkranz JA (2014) Distinct effects of repeated restraint stress on basolateral amygdala neuronal membrane properties in resilient adolescent and adult rats. Neuropsychopharmacology 39:2114–2130. CrossRef Medline

Hopf FW, Bowers MS, Chang SJ, Chen BT, Martin M, Seif T, Cho SL, Tye K, Bonci A (2010) Reduced nucleus accumbens SK channel activity enhances alcohol seeking during abstinence. Neuron 65:682–694. CrossRef Medline

Hopf FW, Seif T, Bonci A (2011a) The SK channel as a novel target for treating alcohol use disorders. Channels (Austin) 5:289–292. CrossRef Medline

Hopf FW, Simms JA, Chang SJ, Seif T, Bartlett SE, Bonci A (2011b) Chlorzoxazone, an SK-type potassium channel activator used in humans, reduces excessive alcohol intake in rats. Biol Psychiatry 69:618–624. CrossRef Medline

Janak PH, Tye KM (2015) From circuits to behaviour in the amygdala. Nature 517:284–292. CrossRef Medline

Kaczorowski CC, Disterhoft J, Spruston N (2007) Stability and plasticity of intrinsic membrane properties in hippocampal CA1 pyramidal neurons: effects of internal anions. J Physiol 578:799–818. CrossRef Medline

Kang MH, Spigelman I, Olsen RW (1998) Alteration in the sensitivity of GABA(A) receptors to allosteric modulatory drugs in rat hippocampus after chronic intermittent ethanol treatment. Alcohol Clin Exp Res 22:2165–2173. CrossRef Medline

Koob GF (2013) Addiction is a reward deficit and stress surfeit disorder. Front Psychiatry 4:72. CrossRef Medline

Koob GF, Le Moal M (2008) Addiction and the brain antireward system. Annu Rev Psychol 59:29–53. CrossRef Medline

Kourrich S, Calu DJ, Bonci A (2015) Intrinsic plasticity: an emerging player in addiction. Nat Rev Neurosci 16:173–184. CrossRef Medline

Kushner MG, Abrams K, Borchardt C (2000) The relationship between anxiety disorders and alcohol use disorders: a review of major perspectives and findings. Clin Psychol Rev 20:149–171. CrossRef Medline

Kye MJ, Spiess J, Blank T (2007) Transcriptional regulation of intronic calcium-

activated potassium channel SK2 promoters by nuclear factor-kappa B and glucocorticoids. Mol Cell Biochem 300:9–17. CrossRef Medline

Lalumiere RT (2014) Optogenetic dissection of amygdala functioning. Front Behav Neurosci 8:107. CrossRef Medline

Lim AL, Taylor DA, Malone DT (2011) Isolation rearing in rats: effect on expression of synaptic, myelin and GABA-related immunoreactivity and its utility for drug screening via the subchronic parenteral route. Brain Res 1381:52–65. CrossRef Medline

Lin MT, Luján R, Watanabe M, Frerking M, Maylie J, Adelman JP (2010) Coupled activity-dependent trafficking of synaptic SK2 channels and AMPA receptors. J Neurosci 30:11726–11734. CrossRef Medline

Lopez MF, Becker HC (2014) Operant ethanol self-administration in ethanol dependent mice. Alcohol 48:295–299. CrossRef Medline

Lowery-Gionta EG, Marcinkiewcz CA, Kash TL (2014) Functional alterations in the dorsal raphe nucleus following acute and chronic ethanol exposure. Neuropsychopharmacology 40:590–600. CrossRef Medline

Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10:434–445. CrossRef Medline

Marcinkiewcz CA, Dorrier CE, Lopez AJ, Kash TL (2014) Ethanol induced adaptations in 5-HT2c receptor signaling in the bed nucleus of stria terminalis: implications for anxiety during ethanol withdrawal. Neuropharmacology 89:157–167. CrossRef Medline

McCool BA, Chappell AM (2009) Early social isolation in male Long-Evans rats alters both appetitive and consummatory behaviors expressed during operant ethanol self-administration. Alcohol Clin Exp Res 33:273–282. CrossRef Medline

McCool BA, Christian DT, Diaz MR, Läck AK (2010) Glutamate plasticity in the drunken amygdala: the making of an anxious synapse. Int Rev Neurobiol 91:205–233. CrossRef Medline

McEwen BS, Olié JP (2005) Neurobiology of mood, anxiety, and emotions as revealed by studies of a unique antidepressant: tianeptine. Mol Psychiatry 10:525–537. CrossRef Medline

Millan MJ (2003) The neurobiology and control of anxious states. Prog Neurobiol 70:83–244. CrossRef Medline

Mulholland PJ (2012) K(Ca)2 channels: novel therapeutic targets for treating alcohol withdrawal and escalation of alcohol consumption. Alcohol 46:309–315. CrossRef Medline

Mulholland PJ, Becker HC, Woodward JJ, Chandler LJ (2011) Small conductance calcium-activated potassium type 2 channels regulate alcohol-associated plasticity of glutamatergic synapses. Biol Psychiatry 69:625–632. CrossRef Medline

Namburi P, Beyeler A, Yorozu S, Calhoon GG, Halbert SA, Wichmann R, Holden SS, Mertens KL, Anahtar M, Felix-Ortiz AC, Wickersham IR, Gray JM, Tye KM (2015) A circuit mechanism for differentiating positive and negative associations. Nature 520:675–678. CrossRef Medline

Oh MM, McKay BM, Power JM, Disterhoft JF (2009) Learning-related postburst afterhyperpolarization reduction in CA1 pyramidal neurons is mediated by protein kinase A. Proc Natl Acad Sci U S A 106:1620–1625. CrossRef Medline

Padula AE, Griffin WC 3rd, Lopez MF, Nimitvilai S, Cannady R, McGuier NS, Chesler EJ, Miles MF, Williams RW, Randall PK, Woodward JJ, Becker HC, Mulholland PJ (2015) KCNN genes that encode small-conductance Ca-activated K channels influence alcohol and drug addiction. Neuropsychopharmacology. Advance online publication. Retrieved March 7, 2015. CrossRef Medline

Pedarzani P, Storm JF (1993) PKA mediates the effects of monoamine transmitters on the K+ current underlying the slow spike frequency adaptation in hippocampal neurons. Neuron 11:1023–1035. CrossRef Medline

Pedersen KA, Schrøder RL, Skaaning-Jensen B, Strøbaek D, Olesen SP, Christophersen P (1999) Activation of the human intermediate-conductance Ca(2+)-activated K(+) channel by 1-ethyl-2-benzimidazolinone is strongly Ca(2+)-dependent. Biochim Biophys Acta 1420:231–240. CrossRef Medline

Ponomarev I, Wang S, Zhang L, Harris RA, Mayfield RD (2012) Gene coexpression networks in human brain identify epigenetic modifications in alcohol dependence. J Neurosci 32:1884–1897. CrossRef Medline

Ren Y, Barnwell LF, Alexander JC, Lubin FD, Adelman JP, Pfaffinger PJ, Schrader LA, Anderson AE (2006) Regulation of surface localization of the small conductance Ca2+-activated potassium channel, Sk2, through direct phosphorylation by cAMP-dependent protein kinase. J Biol Chem 281:11769–11779. CrossRef Medline

Rosenkranz JA, Venheim ER, Padival M (2010) Chronic stress causes amygdala hyperexcitability in rodents. Biol Psychiatry 67:1128–1136. CrossRef Medline

Santini E, Quirk GJ, Porter JT (2008) Fear conditioning and extinction differentially modify the intrinsic excitability of infralimbic neurons. J Neurosci 28:4028–4036. CrossRef Medline

Sellers EM, Zilm DH, Degani NC (1977) Comparative efficacy of propranolol and chlordiazepoxide in alcohol withdrawal. J Stud Alcohol 38:2096–2108. CrossRef Medline

Silberman Y, Bajo M, Chappell AM, Christian DT, Cruz M, Diaz MR, Kash T, Lack AK, Messing RO, Siggins GR, Winder D, Roberto M, McCool BA, Weiner JL (2009) Neurobiological mechanisms contributing to alcohol-stress-anxiety interactions. Alcohol 43:509–519. CrossRef Medline

Silberman Y, Ariwodola OJ, Chappell AM, Yorgason JT, Weiner JL (2010) Lateral paracapsular GABAergic synapses in the basolateral amygdala contribute to the anxiolytic effects of beta 3 adrenoceptor activation. Neuropsychopharmacology 35:1886–1896. CrossRef Medline

Skelly MJ, Weiner JL (2014) Chronic treatment with prazosin or duloxetine lessens concurrent anxiety-like behavior and alcohol intake: evidence of disrupted noradrenergic signaling in anxiety-related alcohol use. Brain Behav 4:468–483. CrossRef Medline

Spanagel R, Montkowski A, Allingham K, Stohr T, Shoaib M, Holsboer F, Landgraf R (1995) Anxiety: a potential predictor of vulnerability to the initiation of ethanol self-administration in rats. Psychopharmacology (Berl) 122:369–373. CrossRef

Stamatakis AM, Sparta DR, Jennings JH, McElligott ZA, Decot H, Stuber GD (2014) Amygdala and bed nucleus of the stria terminalis circuitry: implications for addiction-related behaviors. Neuropharmacology 76:320–328. CrossRef Medline

Stocker M, Pedarzani P (2000) Differential distribution of three Ca2+-activated K+ channel subunits, SK1, SK2, and SK3, in the adult rat central nervous system. Mol Cell Neurosci 15:476–493. CrossRef Medline

Stocker M, Krause M, Pedarzani P (1999) An apamin-sensitive Ca2+-activated K+ current in hippocampal pyramidal neurons. Proc Natl Acad Sci U S A 96:4662–4667. CrossRef Medline

Stuber GD, Sparta DR, Stamatakis AM, van Leeuwen WA, Hardjoprajitno JE, Cho S, Tye KM, Kempadoo KA, Zhang F, Deisseroth K, Bonci A (2011) Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. Nature 475:377–380. CrossRef Medline

Thorsell A, Johnson J, Heilig M (2007) Effect of the adenosine A2a receptor antagonist 3,7-dimethyl-propargylxanthine on anxiety-like and depression-like behavior and alcohol consumption in Wistar Rats. Alcohol Clin Exp Res 31:1302–1307. CrossRef Medline

Tye KM, Janak PH (2007) Amygdala neurons differentially encode motivation and reinforcement. J Neurosci 27:3937–3945. CrossRef Medline

Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature 471:358–362. CrossRef Medline

Washburn MS, Moises HC (1992) Electrophysiological and morphological properties of rat basolateral amygdaloid neurons *in vitro*. J Neurosci 12:4066–4079. Medline

Wright IK, Upton N, Marsden CA (1991) Resocialisation of isolation-reared rats does not alter their anxiogenic profile on the elevated X-maze model of anxiety. Physiol Behav 50:1129–1132. CrossRef Medline

Yorgason JT, España RA, Konstantopoulos JK, Weiner JL, Jones SR (2013) Enduring increases in anxiety-like behavior and rapid nucleus accumbens dopamine signaling in socially isolated rats. Eur J Neurosci 37:1022–1031. CrossRef Medline

Zhang M, Pascal JM, Schumann M, Armen RS, Zhang JF (2012) Identification of the functional binding pocket for compounds targeting small-conductance Ca(2)(+)-activated potassium channels. Nat Commun 3:1021. CrossRef Medline